

Regulation of Type VI Secretion Gene Clusters by σ^{54} and Cognate Enhancer Binding Proteins^{∇†}

Christophe S. Bernard, Yannick R. Brunet, Marthe Gavioli, Roland Llobès, and Eric Cascales*

Laboratoire d'Ingénierie des Systèmes Macromoléculaires Institut de Microbiologie de la Méditerranée Aix-Marseille Université CNRS—UPR9027, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France

Received 7 January 2011/Accepted 19 February 2011

Type VI secretion systems (T6SS) are bacteriophage-derived macromolecular machines responsible for the release of at least two proteins in the milieu, which are thought to form an extracellular appendage. Although several T6SS have been shown to be involved in the virulence of animal and plant pathogens, clusters encoding these machines are found in the genomes of most species of Gram-negative bacteria, including soil, marine, and environmental isolates. T6SS have been associated with several phenotypes, ranging from virulence to biofilm formation or stress sensing. Their various environmental niches and large diversity of functions are correlated with their broad variety of regulatory mechanisms. Using a bioinformatic approach, we identified several clusters, including those of *Vibrio cholerae*, *Aeromonas hydrophila*, *Pectobacterium atrosepticum*, *Pseudomonas aeruginosa*, *Pseudomonas syringae* pv. tomato, and a *Marinomonas* sp., which possess typical $-24/-12$ sequences, recognized by the alternate sigma factor sigma 54 (σ^{54} or σ^N). σ^{54} , which directs the RNA polymerase to these promoters, requires the action of a bacterial enhancer binding protein (bEBP), which binds to *cis*-acting upstream activating sequences. Putative bEBPs are encoded within the T6SS gene clusters possessing σ^{54} boxes. Using *in vitro* binding experiments and *in vivo* reporter fusion assays, we showed that the expression of these clusters is dependent on both σ^{54} and bEBPs.

A large number of macromolecular systems are involved in bacterial pathogenesis. These systems include adhesion factors or organelles required for the secretion of toxin proteins. Among them, type VI secretion systems (T6SS) were first identified in 2006 in a screen for identifying bacterial factors necessary for *Vibrio cholerae* resistance to amoeba predation (40). Since then, T6SS have been characterized or described for many Gram-negative bacteria, including animal and human pathogens (*Pseudomonas aeruginosa*, *Burkholderia* species, pathogenic *Escherichia coli*, *Francisella tularensis*, *Salmonella enterica*), plant pathogens (*Pectobacterium atrosepticum*, *Pseudomonas syringae*, *Agrobacterium tumefaciens*), and fish pathogens (*Edwardsiella tarda*) as well as for soil, marine, and environmental bacteria (2, 15, 32, 36, 38, 42, 45, 48). They are thus widely distributed in a broad spectrum of bacteria in a wide range of environmental niches, but they are also involved in a broad diversity of processes (6, 12, 17, 41, 46, 47). Although necessary for *E. tarda* or *Francisella* pathogenesis, T6SS are required for processes as different as resisting predation in *Vibrio cholerae*, symbiosis in *Rhizobium leguminosarum*, biofilm formation in enteroaggregative *E. coli*, killing of niche competitors in *P. aeruginosa*, *Burkholderia thailandensis*, and *Vibrio cholerae*, and stress sensing in *Vibrio anguillarum* (for a recent review, see reference 46) (2, 7, 21, 30, 34, 40, 47, 60, 66).

This broad variety of environments and functions is reflected

by a large diversity in the regulatory mechanisms: the expression of T6SS gene clusters is usually induced in the presence of host cells, of host cell extracts, or in medium mimicking their environment (5). Several regulatory mechanisms controlling T6SS gene cluster expression have been identified in recent years: they are regulated at the transcriptional level by alternate sigma factors, two-component systems, or transcriptional factors. Several cases of regulation by quorum sensing have also been reported. Because T6SS gene clusters are often found in pathogenicity islands or have been acquired by horizontal gene transfer, their GC content is sometimes different from the GC content of the core genome, and they are silenced by histone-like proteins. T6SS subunit production is also regulated at the translational level through the action of small regulatory RNA, and several T6SS need to be activated by posttranslational mechanisms (for a recent review, see reference 5).

In this paper, we report the characterization of the regulatory mechanism underlying the expression of T6SS gene clusters from *V. cholerae*, *Pectobacterium atrosepticum*, *Aeromonas hydrophila*, and a *Marinomonas* sp. For these clusters, we first identified genes encoding putative bacterial enhancer binding proteins (bEBPs; pfam family PF00158). bEBPs are ATP-dependent activators of the alternate sigma factor σ^{54} (10, 43, 49, 62, 64). σ^{54} (or σ^N), encoded by the *rpoN* gene, recognizes and binds consensus sequences centered at -24 and -12 upstream of the $+1$ transcriptional start. σ^{54} recruits the RNA polymerase (RNAP; E) to these specific promoters, allowing the formation of the closed complex; however, the $E\sigma^{54}$ complex cannot proceed to the open complex, and DNA melting is induced by the energy provided by the bEBP-dependent ATP hydrolysis (10, 62). bEBPs bind at *cis*-acting activating sequences usually located at ~ 100 to 400 bp from the σ^{54} binding

* Corresponding author. Mailing address: Laboratoire d'Ingénierie des Systèmes Macromoléculaires, Institut de Microbiologie de la Méditerranée, Aix-Marseille Université, CNRS—UPR9027, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France. Phone: (33) 491 164 504. Fax: (33) 491 712 124. E-mail: cascales@ifr88.cnrs-mrs.fr.

† Supplemental material for this article may be found at <http://jbb.asm.org/>.

∇ Published ahead of print on 4 March 2011.

box, and they interact with the σ^{54} subunit through DNA bending (facilitated by the integration host factor [IHF]) (62).

Using bioinformatic analyses, we identified putative σ^{54} binding boxes in the promoter regions of the T6SS gene clusters encoding putative bEBPs (see Fig. S1 in the supplemental material). Interestingly, a transposon insertion in the *rpoN* gene has been obtained in the same screen that identified the T6SS gene cluster in *V. cholerae* (40). Recent studies showed that the production of *A. hydrophila* T6SS subunits is abolished in a strain with a deletion of the *vasH* gene, encoding the bEBP (51, 52). However, conflicting data have been recently reported in the case of *V. cholerae*; Ishikawa and colleagues showed that production of the Hcp protein is decreased to undetectable levels in an *rpoN* strain, suggesting that σ^{54} is a positive regulator of the *hcp* gene (22). In contrast, Syed and colleagues reported a negative effect of σ^{54} in the regulatory mechanism of the *V. cholerae* T6SS gene cluster (53). This discrepancy can be probably explained by the difference in the strain background, since the strain used in the latter study is devoid of the *hapR* gene, a transcriptional activator involved in the regulation of the T6SS gene cluster and the *hcp* gene (22, 53). In fact, T6SS are usually subject to complicated regulatory networks that involve transcriptional factors, quorum-sensing molecules and regulators, and various signals (5, 27). Due to cross-talk and feedback loops between these different regulators, indirect effects might occur. To bypass these pathways and avoid indirect regulatory effects, we tested the requirement for σ^{54} and cognate bEBP in T6SS gene cluster regulation in *in vivo* reconstitution assays using the heterologous host model *Escherichia coli*. Using reporter fusion assays, we first show that expression of the *V. cholerae*, *A. hydrophila*, *Pectobacterium atrosepticum*, and *Marinomonas* MWYL1 T6SS gene clusters are controlled by σ^{54} and cognate bEBP. We provide evidence for *in vitro* autophosphorylation of bEBPs and demonstrate binding of σ^{54} and cognate bEBP to the promoter regions by electrophoretic mobility shift assays (EMSAs).

MATERIALS AND METHODS

Bacterial strains, chemicals, media, and growth conditions. The *Escherichia coli* strain W3110 and its *uidA* and *rpoN* derivatives were used throughout the study. The *E. coli* DH5 α strain was used for cloning procedures. BL21(DE3) was used for protein production. The *E. coli*, *Vibrio cholerae* O395, and *Aeromonas hydrophila* ATCC7966 strains were grown in LB medium at 37°C. *Pectobacterium atrosepticum* SCRI11043 was grown in LB medium at 30°C, whereas *Marinomonas* sp. MWYL-1 was grown in LB medium supplemented with 5% sodium chloride at 30°C. Genomic DNA from these strains was prepared using a DNeasy blood and tissue kit (Qiagen). Acetyl-phosphate and *para*-nitrophenyl- β -D-glucuronide were purchased from Sigma-Aldrich. Purified RNAP core enzyme was obtained from Epicentre Biotechnology. [32 P]Orthophosphate (phosphorus) was purchased from PerkinElmer.

Strain construction. The W3110 *uidA::Kan^r* strain was obtained by P1 bacteriophage transduction from BW25113 *uidA::Kan^r* (Keio collection) (4). The kanamycin cassette was then excised using the pCP20 plasmid as described previously (14) to yield W3110 Δ *uidA*. The W3110 Δ *uidA* Δ *rpoN* strain was obtained similarly, by P1 transduction from the BW25113 *rpoN::Kan^r* strain and cassette excision.

Plasmid construction. PCRs were performed with a Biometra thermocycler, using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA). Plasmids (listed in Table 1) were constructed by a double PCR technique, allowing amplification of the gene or promoter of interest flanked by extensions annealing to the target vector (3, 58). The products of the first PCR were then used as oligonucleotides for a second PCR using the target vector as the template (custom oligonucleotides, synthesized by Eurogentec, are listed in Table 2). For *uidA* transcriptional fusions, the promoter sequences were amplified from genomic DNA and cloned

downstream of the T7 promoter into the pT7.7 vector (54). The *uidA* gene, encoding β -glucuronidase, has then been cloned downstream of each promoter. For bEBP-producing plasmids, the gene encoding the bacterial enhancer binding protein fused to an N-terminal FLAG epitope (DYKDDDDK) has been amplified from genomic DNA and cloned into the pOK12 vector (59) downstream of the *lac* promoter. For protein purification, genes encoding the *E. coli* σ^{54} protein and the various bEBPs were amplified from genomic DNA and cloned into the pET19b plasmid (Novagen). The production of the protein fused to an N-terminal 10-histidine tail is then controlled by the T7 promoter. All constructs were verified by restriction analyses and DNA sequencing (Genome Express).

β -Glucuronidase assays. β -Glucuronidase enzyme activity was measured from cells in mid-exponential phase (optical density at 600 nm [OD₆₀₀] of 0.6) as previously described (23). Briefly, 0.2 ml of cell culture was mixed with 0.8 ml of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄ [pH 7.0], 10 mM KCl, 1 mM MgSO₄, 10 mM β -mercaptoethanol, 0.1% Triton X-100) and lysed by the addition of one drop of 0.1% SDS and two drops of chloroform. After vigorous vortexing, the cell extracts were diluted four times in Z buffer, and β -glucuronidase activity (release of *para*-nitrophenol) was monitored at 405 nm after the addition of *para*-nitrophenyl- β -D-glucuronide (Sigma-Aldrich; 10 mM final concentration). The specific β -glucuronidase activity was expressed in Miller units. Each enzymatic assay was performed in triplicate, starting from each biological triplicate (from independent plasmid transformations).

Protein purification. The *E. coli* σ^{54} protein and the bEBPs from the various species were purified as follows. Proteins were produced in BL21(DE3) carrying the corresponding pET19b derivative, by T7 polymerase gene induction using 100 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) for 16 h at 22°C. Despite optimization of the production conditions, bEBPs always remained in inclusion bodies. bEBPs were therefore solubilized and purified in the presence of urea as described below. Cells were centrifuged at 20,000 \times g for 20 min, and the pellet was resuspended in 20 mM Tris-HCl (pH 8.0)–100 mM NaCl (TN buffer) supplemented with protease inhibitors (Complete EDTA-free; Roche) and 8 M urea. Cells were then lysed by the addition of lysozyme (100 μ g/ml) and the use of a French press. After removal of insoluble material by centrifugation for 45 min at 90,000 \times g, histidine-tagged proteins were immobilized on an ion metal affinity chromatography resin (Cobalt Talon resin; Clontech) preequilibrated in TN buffer supplemented with urea. Proteins were eluted by using an imidazole gradient, and the fractions containing concentrated and pure proteins were pooled and dialyzed stepwise against TN buffer containing 6 M, 4 M, and 2 M urea and no urea. At this step, a large portion of the bEBPs precipitated and were removed by ultraspeed centrifugation (15 min at 30,000 \times g). The Fur protein was purified from plasmid pBT4-1 (55) as previously published (55). The Fur protein was shown to be functional in an electrophoretic mobility shift assay using the Fur-dependent *Escherichia coli* K-12 *cirA* promoter as the probe (data not shown). The purity of each protein was estimated to be >95% based on Coomassie blue staining. The concentration of each protein was determined by the absorbance at 280 nm using theoretical molar extinction coefficients calculated using an ExPASy website tool (<http://www.expasy.ch/tools/protparam.html>).

Electrophoretic mobility shift assays (EMSAs). 32 P-Labeled probes were obtained by PCR amplification of the promoter sequences from chromosomal DNA using dNTP mixes containing [α - 32 P]deoxyadenosine triphosphate. Labeled probes were column purified (Wizard SV gel and PCR cleanup kit; Promega) to remove radioactive nucleotides. Gel shift experiments were carried out with soluble bEBPs and with reconstituted E σ^{54} . DNA binding activity of E σ^{54} was measured as previously described (11, 50, 56). Briefly, 32 P-labeled probes were mixed with E σ^{54} (premixed as an RNAP core enzyme at a σ^{54} ratio of 1:2) in STA buffer (25 mM Tris-acetate [pH 8.0], 8 mM magnesium acetate, 10 mM KCl, 1 mM dithiothreitol [DTT], and 3.5% PEG 6000) in the presence of sonicated salmon sperm DNA (10 μ g/ml) and bovine serum albumin (BSA; 50 μ g/ml). Controls include incubation with RNAP core enzyme or with double-stranded DNA competitors (consensus σ^{54} and Fur binding boxes obtained by the annealing of two complementary oligonucleotides; Table 2). Binding of bEBPs to the promoters was tested by adaptation of previously described protocols (13, 24, 57). Purified bEBPs were mixed with 32 P-labeled promoter probes in a binding reaction mixture containing sonicated salmon sperm DNA (10 μ g/ml), BSA (100 μ g/ml), 5 mM MgCl₂, 50 mM potassium acetate, 5% glycerol, 1 mM DTT, and 10 mM acetyl-phosphate in 25 mM Tris-HCl (pH 7.9). After the 20-min binding step, samples were resolved using a prerun 9% acrylamide gel in Tris-borate buffer. Gels were fixed in 10% trichloroacetic acid for 10 min and exposed to Kodak BioMax MR films.

***In vitro* phosphorylation.** Acetyl- [32 P]phosphate was synthesized as previously described (33). Phosphorylation assays were performed as previously described (16, 29) with minor modifications. Briefly, 10 μ g of protein was incubated for 30

TABLE 1. Plasmids constructed for this study

Plasmid category (vector) ^a and name	Description
<i>uidA</i> transcriptional fusion (pT7.7)	
pP _{glnA}	<i>Escherichia coli glnA</i> (GenBank accession no. AP_003938) promoter (379 bp, starting at position -376 from the AUG start codon) upstream of the <i>uidA</i> gene
pP _{VCA0107}	<i>Vibrio cholerae</i> type VI secretion gene cluster (VCA0107; accession no. NP_232508) promoter (390 bp, starting at position -387 from the AUG start codon) upstream of the <i>uidA</i> gene
pP _{VCA0017}	<i>Vibrio cholerae hcp-vgrG</i> operon (VCA0017; accession no. NP_232418) promoter (397 bp, starting at position -394 from the AUG start codon) upstream of the <i>uidA</i> gene
pP _{ECA3445}	<i>Pectobacterium atrosepticum</i> type VI secretion gene cluster (ECA3445; accession no. YP_051535) promoter (604 bp, starting at position -601 from the AUG start codon) upstream of the <i>uidA</i> gene
pP _{ECA2866}	<i>Pectobacterium atrosepticum hcp-vgrG</i> operon (ECA2866; accession no. YP_050957) promoter (326 bp, starting at position -323 from the AUG start codon) upstream of the <i>uidA</i> gene
pP _{ECA4275}	<i>Pectobacterium atrosepticum hcp-vgrG</i> operon (ECA4275; accession no. YP_052352) promoter (730 bp, starting at position -727 from the AUG start codon) upstream of the <i>uidA</i> gene
pP _{AHA1826}	<i>Aeromonas hydrophila</i> type VI secretion gene cluster (AHA_1826; accession no. YP_856360) promoter (606 bp, starting at position -603 from the AUG start codon) upstream of the <i>uidA</i> gene
pP _{MWYL1196}	<i>Marinomonas</i> sp. MWYL1 type VI secretion gene cluster, operon 1 (<i>Mar-1</i>) (Mmwyl1_1196; accession no. YP_001340060) promoter (744 bp, starting at position -741 from the AUG start codon) upstream of the <i>uidA</i> gene
pP _{MWYL1195}	<i>Marinomonas</i> sp. MWYL1 type VI secretion gene cluster, operon 2 (<i>Mar-2</i>) (Mmwyl1_1195; accession no. YP_001340059) promoter (744 bp, starting at position -741 from the AUG start codon) upstream of the <i>uidA</i> gene
Epitope-tagged EBP production (pOK12)	
pOK-NtrC.....	<i>Escherichia coli ntrC</i> gene (accession no. AP_003940)
pOK-VCA0117.....	<i>Vibrio cholerae vasH</i> gene (VCA0117; accession no. NP_232518)
pOK-ECA3435.....	<i>Pectobacterium atrosepticum vasH</i> gene (ECA3435; accession no. YP_051525)
pOK-AHA1842.....	<i>Aeromonas hydrophila vasH</i> gene (AHA_1842; accession no. YP_856376)
pOK-MWYL1206.....	<i>Marinomonas</i> sp. MWYL1 <i>vasH</i> gene (Mmwyl1_1206; accession no. YP_001340070)
Protein purification (pET19b)	
pET-RpoN.....	<i>Escherichia coli rpoN</i> gene (accession no. AP_003745)
pET-NtrC.....	<i>Escherichia coli ntrC</i> gene (accession no. AP_003940)
pET-VCA0117.....	<i>Vibrio cholerae vasH</i> gene (VCA0117; accession no. NP_232518)
pET-ECA3435.....	<i>Pectobacterium atrosepticum vasH</i> gene (ECA3435; accession no. YP_051525)
pET-AHA1842.....	<i>Aeromonas hydrophila vasH</i> gene (AHA_1842; accession no. YP_856376)
pET-MWYL1206.....	<i>Marinomonas</i> sp. MWYL1 <i>vasH</i> gene (Mmwyl1_1206; accession no. YP_001340070)

^a Plasmids within each category were cloned in the indicated vector (shown in parentheses) for that category.

min in 40 mM Tris-HCl (pH 7.2) in the presence of 5 mM MgCl₂, 1 mM DTT, and 5 mM acetyl-[³²P]phosphate. The reaction was quenched by the addition of loading buffer, and the proteins were subjected to electrophoresis using a 10% SDS-PAGE, transferred onto nitrocellulose membrane, autoradiographed, and then immunodetected with anti-His monoclonal antibody.

Miscellaneous. For detection by immunostaining, proteins were transferred onto nitrocellulose membranes, and blots were probed with anti-PentaHis (Qiagen) or M2 anti-FLAG (Sigma-Aldrich) monoclonal antibodies and anti-mouse secondary antibodies coupled to alkaline phosphatase and developed using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

RESULTS

Identification of σ^{54} binding boxes. To identify T6SS gene clusters potentially controlled by σ^{54} , we performed a survey using the BProm algorithm (SoftBerry). The putative σ^{54} -dependent T6SS promoter list includes T6SS gene clusters from *Vibrio cholerae*, *Pectobacterium atrosepticum*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa* (HSI-2 and HSI-3), *P. syringae* pv. tomato (two clusters), and a *Marinomonas* sp. (Fig. 1A) (see Fig. S1 in the supplemental material). These promoters

were found upstream of the first gene of the cluster, suggesting that all the genes that are organized in the same orientation are part of an operon structure. In one case (*Pectobacterium atrosepticum*), an additional promoter was identified upstream of the ECA3428 gene, suggesting that the cluster is composed of two operons (see Fig. S1). In the case of T6SS, genes are present within two divergent operons, and a σ^{54} binding sequence is found in both orientations (*P. aeruginosa* HSI-3 and the *Marinomonas* sp.) (see Fig. S1). Interestingly, genes encoding putative bEBPs are present within T6SS gene clusters that share putative σ^{54} boxes (see Fig. S1).

Activities of transcriptional fusions. To test whether the type VI secretion gene clusters that share putative σ^{54} binding sequences are directly regulated by σ^{54} , we used an *in vivo* reconstitution approach into the heterologous host *Escherichia coli*. This allowed reducing indirect regulatory effects by other factors, cross-talk, regulatory feedback loops, and growth conditions. We constructed transcriptional fusions, in which the *uidA* gene, encoding β -glucuronidase, is under the control

TABLE 2. Oligonucleotides used in this study

Oligonucleotide purpose and name	Sequence
Promoter insertion in pT7.7^a and gel shift expts	
<i>P_{ghnA}</i>	<u>CTCACTATAGGGAGACCGGAATTCGAGCTCGCCTCAGGCATTAGAAATAGCGCGTTATTG</u> GGTCGACTCTAGAGGATCCCCGGGCCATACITTAACCTCTCCTGGATTGGTCATGGTC
<i>P_{VCA0107}</i>	<u>CTCACTATAGGGAGACCGGAATTCGAGCTCGGGGTAAGATCACGCTTCGGG</u> GGTCGACTCTAGAGGATCCCCGGGCCATATTACGTCTCCAATACCTATGCCAAACG
<i>P_{VCA0017}</i>	<u>CTCACTATAGGGAGACCGGAATTCGAGCTCTCCCGTTTGTGCGTTATACCTTTCCTAC</u> GGTCGACTCTAGAGGATCCCCGGGCTATTTCTTTCAATAAATCATTTTAAAGTCAATG
<i>P_{ECA3445}</i>	<u>CTCACTATAGGGAGACCGGAATTCGAGCTCCCTTAATTTGTAGCGGGTTAAGTATTTCTGTT</u> GGTCGACTCTAGAGGATCCCCGGGCCATGTGTCTATACCAACGTAAGGGCAGCTCG
<i>P_{ECA2866}</i>	<u>CTCACTATAGGGAGACCGGAATTCGAGCTCTGATATTGATAAAATTTATGAGGATATCCTCCCC</u> ATTC GGTCGACTCTAGAGGATCCCCGGGCAGTCTTGCTCCTTGTGTTGAACGTGATG
<i>P_{ECA4275}</i>	<u>CTCACTATAGGGAGACCGGAATTCGAGCTCACCGCAAGTATCAGCAATAAGTGTTC</u> GGTCGACTCTAGAGGATCCCCGGGCGGAGTTGGCATAGTGTGCTCCTGTGTTG
<i>P_{AHA1826}</i>	<u>CTCACTATAGGGAGACCGGAATTCGAGCTCGGAAGGGCAGGGAACATAGAGCC</u> GGTCGACTCTAGAGGATCCCCGGGCCATGGGTATGCTCCTGATTTGAACG
<i>P_{MWYL1196 (Mar-1)}</i>	<u>CTCACTATAGGGAGACCGGAATTCGAGCTCATACTTTCTCCATCCAAACGACGTC</u> GGTCGACTCTAGAGGATCCCCGGGCATAGTAAAATTCCTTATAAAAATAGTAAAAACGTG
<i>P_{MWYL1195 (Mar-2)}</i>	<u>CTCACTATAGGGAGACCGGAATTCGAGCTCATAGTAAAATTCCTTATTTAAAAATAGTAAAAA</u> CGTG GGTCGACTCTAGAGGATCCCCGGGCATACITTTCTCCATCCAAACGACGTC
<i>uidA</i>	GCGCGGGGATCCTCTAGAGTGCACCATGTTACGTCCTGTAGAACCACCCCAACCCG <u>ACAGCTTATCATCGATAAGCTTGGGCTGCATCATTTGTTGCTCCCTGCTGCGG</u>
EBP insertion in pOK12^{a,b}	
NtrC	<u>GGATAACAATTTACACAGGAAACAGCTATGGACTACAAAGACGACGATGACAAGCAACGAGG</u> GATAGTCTGGGTAGTCG GGCCTCGACTAGTGGCGTAATCATGGTCACTCCATCCCAGCTCTTTAACTTAC
<i>VCA0117</i>	<u>GGATAACAATTTACACAGGAAACAGCTATGGACTACAAAGACGACGATGACAAGAGTCAATGG</u> CTGGCGTTTGCAACC GGCCTCGACTAGTGGCGTAATCATGGTCACTGGGGTTTGTATCTCCAATTTCAAG
<i>ECA3435</i>	<u>GGATAACAATTTACACAGGAAACAGCTATGGACTACAAAGACGACGATGACAAGCAACATGCC</u> CTCAAACCTGGCGCTAG GGCCTCGACTAGTGGCGTAATCATGGTCAATTCACCTCCAGCTTCTGGCATTG
<i>AHA1842</i>	<u>GGATAACAATTTACACAGGAAACAGCTATGGACTACAAAGACGACGATGACAAGGAGCAAGC</u> CCTCGCATTTTCCCTG GGCCTCGACTAGTGGCGTAATCATGGTCACTCCAGTTTCTGGCACTTG
<i>MWYL1206</i>	<u>GGATAACAATTTACACAGGAAACAGCTATGGACTACAAAGACGACGACGATAAATGGCTTAAG</u> TCTGCAGCTGAGTTGG <u>GGCCTCGACTAGTGGCGTAATCATGGTCTAATCATTTATGAAATCTCCAATTTCAAACAC</u>
RpoN and EBP insertion in pET19b^c	
RpoN	<u>GCGGCCATATCGACGACGACGACAAGAAGCAAGGTTTGCACTCAGGCTTAGCC</u> GTTAGCAGCCGATCCTCGAGCATATGTCAAACGAGTTGTTACGCTGGTTTGACG
NtrC	<u>GGCCATATCGACGACGACGACAAGCAACGAGGGATAGTCTGGGTAGTCTGATG</u> GCAGCCGATCCTCGAGCATATGTCACTCCATCCCAGCTCTTTAACTTAC
<i>VCA0117</i>	<u>GGCCATATCGACGACGACGACAAGAGTCAATGGCTGGCGTTTGCAACC</u> GCAGCCGATCCTCGAGCATATGTCACTGGGTTTGTATCTCCAATTTCAAG
<i>ECA3435</i>	<u>GGCCATATCGACGACGACGACAAGCAACATGCCCTCAAACCTGGCGCTAG</u> GCAGCCGATCCTCGAGCATATGTCAATTCACCTCCAGCTTCTGGCATTG
<i>AHA1842</i>	<u>GGCCATATCGACGACGACGACAAGAGCAAGCCCTCGCATTTTCCCTG</u> GCAGCCGATCCTCGAGCATATGTCACTGAGTTCACTCCAGTTTCTGGCACTG
<i>MWYL1206</i>	<u>GGCCATATCGACGACGACGACAAGAATAACTGGCTTAAGTCTGCAGCTGAGTTGGT</u> GCAGCCGATCCTCGAGCATATGCTAATCATTTATGAAATCTCCAATTTCAAACAC
Consensus boxes for competition expts^c	
RpoN	<u>TATGCCGAAGGGTGGCAGGATGATTGCATATGCCG</u> CGGCATATGCAATCATCGTGCCACCCTTCGGCATA
Fur	<u>TATGCCGATAATGATAATCATTATCTATGCCG</u> CGGCATAGATAATGATTATCATTATCCGGCATA

^a Sequence complementary to the target vector is underlined.

^b The FLAG tag coding sequence is italicized.

^c The consensus sequence is underlined.

of the promoter region of the type VI secretion gene cluster of *Vibrio cholerae* (*P_{VCA0107}*), *Pectobacterium atrosepticum* (*P_{ECA3445}*), or *Aeromonas hydrophila* (*P_{AHA1826}*). The *Mariomonas* sp. T6SS genes are distributed in two distinct divergent operons, and σ^{54} binding sequences are found in both orientations in the promoter region; the region between these two operons has been thus cloned in both orientations (*P_{MWYL1195}* and *P_{MWYL1196}*). Plasmids carrying the transcriptional fusion have been introduced into the *E. coli uidA* strain or its *rpoN* derivative, and β -glucuronidase activities have been

measured. Figure 2A shows that the fusions have a low level of activity in both backgrounds.

σ^{54} binds to type VI secretion gene cluster promoters. We purified the *E. coli* σ^{54} protein and tested its ability to interact with the T6SS gene cluster promoters in an electrophoretic mobility shift assay (EMSA). Labeled promoters were incubated with increasing concentrations of $\text{E}\sigma^{54}$. Several specific controls were included, such as (i) incubation with the RNAP core enzyme (i.e., devoid of σ^{54}), (ii) the use of a specific competitor consisting of a consensus *E. coli* σ^{54} binding se-

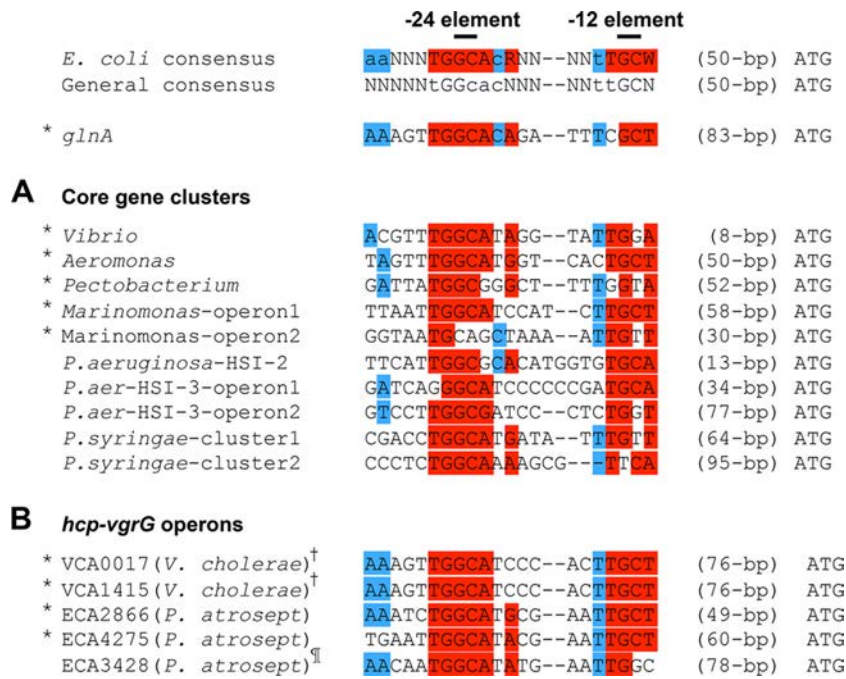


FIG. 1. Alignment of putative σ^{54} binding sequences within T6SS gene cluster promoters. Putative σ^{54} promoters identified for T6SS gene clusters (A) or orphan *hcp* and *vgrG* genes (B) characterized or discussed in this study are aligned using Clustal W with the *E. coli* σ^{54} binding sequence consensus (upper line) (44). The promoters of the T6SS gene clusters are P_{VCA0107} (*Vibrio cholerae*), P_{ECA3445} (*Pectobacterium atrosepticum* [*P. atrosept*]), P_{AHA1826} (*Aeromonas hydrophila*), P_{MWYL1196} (*Marinomonas* sp., operon 1), P_{MWYL1195} (*Marinomonas* sp., operon 2), P_{PA1656} (*Pseudomonas aeruginosa* HSI-2), P_{PA2365} (*P. aeruginosa* HSI-3, operon 1), P_{PA2364} (*P. aeruginosa* HSI-3 [*P. aer*-HSI-3], operon 2), P_{PSPTO2541} (*P. syringae*, cluster 1), P_{PSPTO5428} (*P. syringae*, cluster 2) (see Fig. S1 in the supplemental material for promoter location details). Base conservation in the *E. coli* consensus is indicated with a color code (red, highly conserved; blue, partly conserved; white, variable base). Promoters characterized in this study are indicated by asterisks. † indicates that these two promoters (VCA0017 and VC1415) are strongly similar (two mismatches along a 200-bp sequence). The region upstream of the ECA3428 gene (encoding an *hcp* homologue in the main *Pectobacterium* T6SS gene cluster) also contains a putative σ^{54} promoter (indicated by ¶).

quence (tatgccgAAgggTGGCACgatgaTTGCATatgccg; the consensus sequence is in uppercase) (44), (iii) the use of a nonspecific competitor corresponding to a consensus *E. coli* Fur binding sequence (tatgccgGATAATGATAATCATTATC tatgccg; the consensus sequence is in uppercase) (55), and (iv) the incubation of E σ^{54} with the promoter region of the enteroaggregative *E. coli* *sci1* T6SS gene cluster, which shares a typical σ^{70} binding sequence. Figure 3 shows that the promoter regions of the various T6SS gene clusters are retarded in the presence of increasing amounts of E σ^{54} and that the mobility shifts are abolished in the presence of an excess of the specific cold competitor. Further, the *Marinomonas* promoter displays two retarded bands (Fig. 3), a result compatible with the presence of two putative σ^{54} binding boxes regulating the two divergent operons. Taken together, the data reported in Fig. 3 suggest that the promoter regions of these T6SS gene clusters are under the control of σ^{54} .

Activities of the transcriptional fusions are dependent on the bEBP. Activity of σ^{54} -dependent promoter relies on transcriptional activators of the EBP family. We therefore tested whether the bacterial EBPs encoded by the *vasH* genes enhanced the activities of the transcriptional fusions. The *vasH* genes from *V. cholerae*, *Pectobacterium atrosepticum*, *A. hydrophila*, and the *Marinimonas* sp. were cloned in the pOK12 vector under the control of the *lac* promoter and fused to an N-terminal FLAG epitope coding sequence. Western blot

analyses showed that all the bEBPs (VasH) were produced (Fig. 2B, inset). pOK12 is a P15A replicon derivative and thus is compatible with the vector carrying the β -glucuronidase reporter fusions. Figure 2B shows that overproduction of the cognate bEBP increases the activity of the transcriptional fusions of the different T6SS gene clusters 20- to 30-fold. This high expression level is dependent upon the presence of the σ^{54} alternate factor, since activities of the fusions in the presence of bEBPs are abrogated in the *rpoN* mutant strain (Fig. 2B). As a control, the activity of the enteroaggregative *sci1* T6SS promoter was shown to be independent of σ^{54} and of the *E. coli* NtrC proteins (data not shown). These data confirm that the expression of the *V. cholerae*, *Pectobacterium atrosepticum*, *A. hydrophila*, and *Marinimonas* T6SS gene clusters is under the control of σ^{54} and further show that cognate bEBPs are required for activation of transcription.

bEBP autophosphorylation. *In vivo*, response regulators are phosphorylated by cognate sensor kinases. *In vitro*, these response regulators are often capable of autophosphorylation in the presence of small-molecule phosphodonors, such as acetylphosphate or carbamyl-phosphate. This characteristic has also been reported for bEBPs, including NtrC (16, 29, 33, 61). We therefore tested whether the T6SS-associated bEBPs are capable of autophosphorylation. *E. coli* NtrC and the different T6SS bEBPs were purified to homogeneity by ion metal affinity chromatography after solubilization of inclusion bodies with

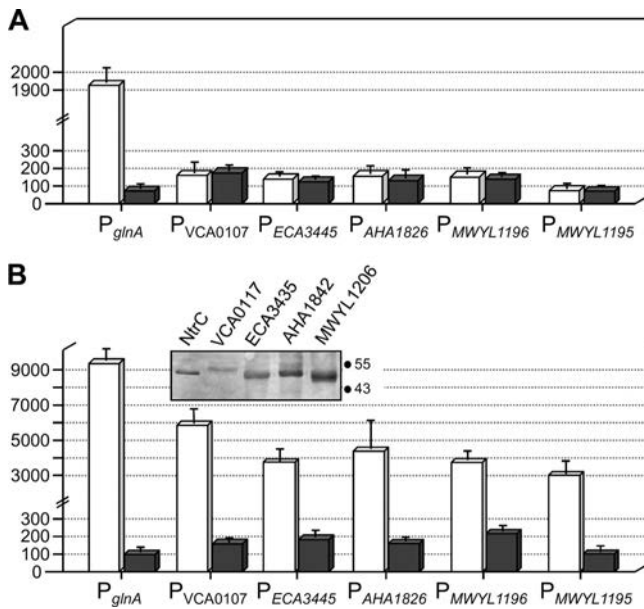


FIG. 2. T6SS gene clusters are regulated by σ^{54} and cognate enhancer binding proteins. β -Glucuronidase activity of transcriptional fusions in the *Escherichia coli* wild type strain (white bars) and its *rpoN* derivative strains (black bars), in the absence (A) or presence (B) of bEBP ectopic overproduction. The inset in panel B shows the levels of NtrC and T6SS-associated bEBPs produced during the experiments: VCA0117 (*V. cholerae*), ECA3435 (*Pectobacterium atrosepticum*), AHA1842 (*A. hydrophila*) and MWYL1206 (*Marinomonas* sp.) (immunodetected by the anti-FLAG monoclonal antibody); molecular mass markers (in kilodaltons) are at the right.

urea (see Materials and Methods). Soluble bEBPs were then incubated in the presence of a low-molecular-mass phosphodonator. Figure 4 shows that the bEBPs from the *V. cholerae*, *Pectobacterium atrosepticum*, *A. hydrophila*, and *Marinomonas* sp. T6SS were phosphorylated in the presence of acetyl- $[^{32}P]$ phosphate, as was NtrC. As the control, the σ^{54} protein was not phosphorylated under the same conditions.

Binding of the σ^{54} -dependent activator on type VI secretion gene cluster promoters. The results of the reporter fusion assays (Fig. 2B) suggest that the bEBPs bind to the T6SS gene cluster promoters to activate their transcription. To test this hypothesis, we performed EMSA using the purified bEBPs. As shown in Fig. 5, the bEBPs specifically bind the promoter region of their cognate T6SS gene cluster. As controls, (i) the purified Fur protein did not retard the different promoters, and (ii) the various bEBPs did not interact with the enteroaggregative *E. coli sci-1* promoter.

Orphan *hcp* and *vgrG* genes are coregulated with the core T6SS gene cluster. Our bioinformatic survey indicated that σ^{54} binding sequences are present in the promoter region of genes encoding Hcp and VgrG subunits when those are isolated elsewhere on the chromosome (Fig. 1B) (see Fig. S1 in the supplemental material). The promoter regions of the orphan *hcp-vgrG* operons from *V. cholerae* ($P_{VCA0017}$) and *Pectobacterium atrosepticum* ($P_{ECA2866}$ and $P_{ECA4275}$) were then cloned and tested for their σ^{54} and bEBP dependence. Reporter fusion studies demonstrated that the expression of these genes

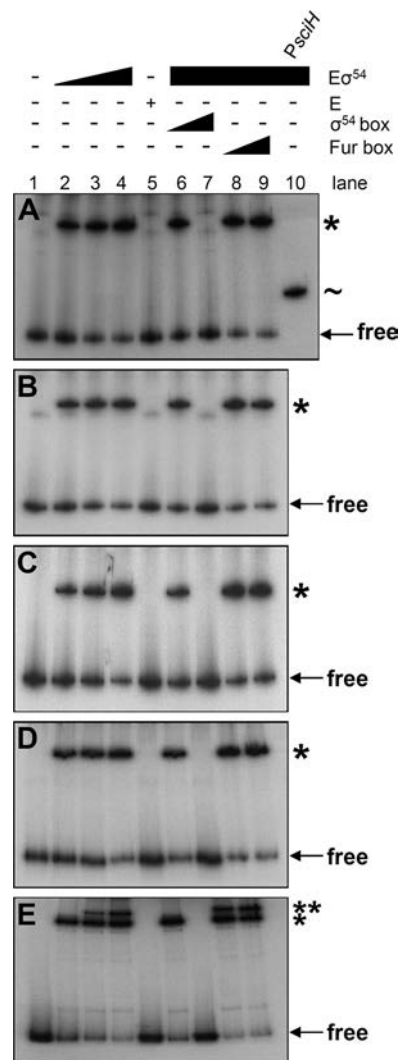


FIG. 3. σ^{54} binds to T6SS gene cluster promoters. Gel shift assays using reconstituted σ^{54} -RNA polymerase complex ($E\sigma^{54}$) (lane 1, no protein; lane 2, 25 nM; lane 3, 100 nM; lane 4, 250 nM) or RNA polymerase core enzyme (E) (lane 5; 250 nM). Competition experiments using double-stranded consensus σ^{54} binding box (lane 6, molecular ratio [probe/competitor] of 1:5; lane 7, molecular ratio of 1:25) or nonspecific consensus Fur binding box (lane 8, molecular ratio [probe/competitor] of 1:5; lane 9, molecular ratio of 1:25). (A) *E. coli glnA* promoter; (B) *V. cholerae* T6SS gene cluster promoter ($P_{VCA0107}$); (C) *Pectobacterium atrosepticum* T6SS gene cluster promoter ($P_{ECA3445}$); (D) *A. hydrophila* T6SS gene cluster promoter ($P_{AHA1826}$); (E) *Marinomonas* MWYL1 T6SS gene cluster promoter ($P_{MWYL1196}/P_{MWYL1195}$). Retarded probe- $E\sigma^{54}$ complexes are indicated by asterisks. Control with the enteroaggregative *E. coli sci1* T6SS gene cluster promoter probe (P_{sci1} ; indicated by ~) is shown (panel A, lane 10, 250 nM). free, labeled promoter probe.

and operons is controlled by σ^{54} and by the bEBP encoded within the T6S core cluster (Fig. 6A and B). This result was validated by gel shift assays showing that the promoter regions of VCA0017, ECA2866, and ECA4275 are retarded by σ^{54} (Fig. 6C) and bEBP (Fig. 6D). Together, these results show that orphan *hcp* and *vgrG* genes are coregulated with the main T6SS gene cluster.

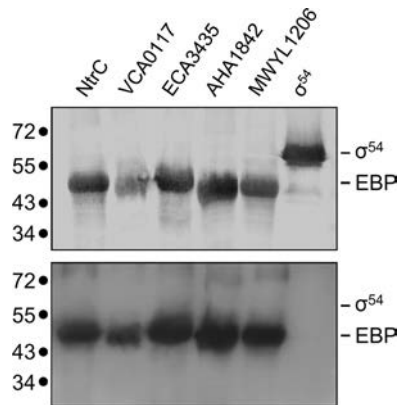


FIG. 4. T6SS-associated bEBPs autophosphorylate. Purified bEBPs from *E. coli* (NtrC, lane 1), or from *V. cholerae* (VCA0117; lane 2), *Pectobacterium atrosepticum* (ECA3435; lane 3), *A. hydrophila* (AHA1842; lane 4), or *Marinomonas* (MWYL1206; lane 5) T6SS gene clusters and the *E. coli* σ^{54} protein (lane 6), incubated with the low-molecular-weight phosphodonor [32 P]acetyl-phosphate were separated by 10% acrylamide SDS-PAGE, blotted, and immunodetected by anti-His antibody (upper panel) or detected by autoradiography (lower panel). Molecular masses (in kilodaltons) are indicated on the left.

DISCUSSION

In this work, we used an *in vivo* reconstitution approach in the heterologous host *E. coli* and *in vitro* DNA binding assays to study the role of the alternate sigma factor σ^{54} in the regulation of several T6SS gene clusters. This allowed us to bypass other regulatory determinants and to specifically study σ^{54} -dependent regulation and the role of bEBP encoded within the T6SS gene clusters. Our results from the reporter fusions show that the bEBP from several microorganisms, such as *V. cholerae*, *A. hydrophila*, *Pectobacterium atrosepticum*, and *Marinomonas* sp., adapt with the *E. coli* σ^{54} -RNAP complex. Interactions of a bEBP and a σ^{54} from two distinct microorganisms have already been demonstrated (50). This is made possible by the high degree of conservation between the σ^{54} binding region of *E. coli* bEBPs and the bEBPs used in this study: the central domain, including the GAFTGA sequence, which mediates bEBP binding to σ^{54} , is conserved among all the bEBPs used in this study (8, 43, 49, 65) (see Fig. S2 in the supplemental material). Reciprocally, the σ^{54} region I (residues 15 to 47), which is involved in bEBP recognition, is highly conserved between the *E. coli* σ^{54} and the σ^{54} subunits from the various microorganisms used in this study (11, 63). However, although these specific sequences are conserved among the bEBPs, a sequence alignment between the bEBPs studied in this work and NtrC showed that the N-terminal activation domain and the C-terminal DNA binding domain are the less-conserved regions (see Fig. S2 in the supplemental material). This observation suggests that the T6SS-associated bEBPs respond to different signals and bind different DNA sequences.

Using a combination of reporter fusion and gel shift experiments, we conclude that the expression of the *V. cholerae*, *Pectobacterium atrosepticum*, *A. hydrophila*, and *Marinomonas* sp. T6SS gene clusters are positively controlled by σ^{54} and cognate bEBPs. Our results confirm and extend previous studies showing that (i) σ^{54} and the VasH bEBP are required for expression of the *V. cholerae* T6SS gene cluster (40), (ii) σ^{54} is

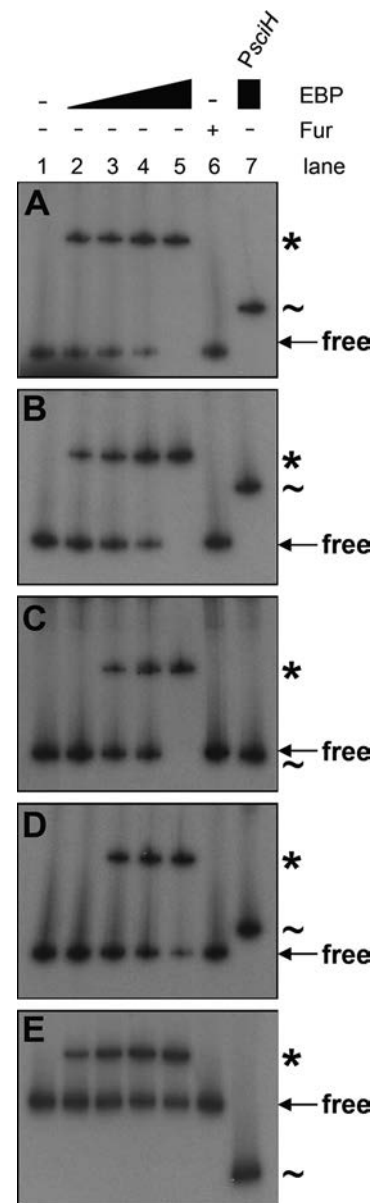


FIG. 5. bEBPs bind to T6SS gene cluster promoters. Gel shift assays using purified bEBPs (lane 1, no protein; lane 2, 50 nM; lane 3, 200 nM; lane 4, 400 nM; lane 5, 800 nM) or a specificity control using the purified Fur protein (lane 6, 800 nM). (A) *E. coli* *glnA* promoter; (B) *V. cholerae* T6SS gene cluster promoter ($P_{VCA0107}$); (C) *Pectobacterium atrosepticum* T6SS gene cluster promoter ($P_{ECA3445}$); (D) *A. hydrophila* T6SS gene cluster promoter ($P_{AHA1826}$); (E) *Marinomonas* MWYL1 T6SS gene cluster promoter ($P_{MWYL1196}/P_{MWYL1195}$). Retarded probe-bEBP complexes are indicated by asterisks. Control with the enteroaggregative *E. coli* *scil* T6SS gene cluster promoter probe (P_{scil} ; indicated by ~) and each bEBP protein is shown (lane 7, 800 nM).

necessary for *V. cholerae* Hcp production (22), and (iii) the *A. hydrophila* *vasH* gene is necessary for T6SS production (51). In the case of the *Marinomonas* sp., the cluster is distributed into two divergent operons, under the control of a single intergenic sequence which contains two divergent σ^{54} promoters but probably with a unique binding site for the bEBP. Expression

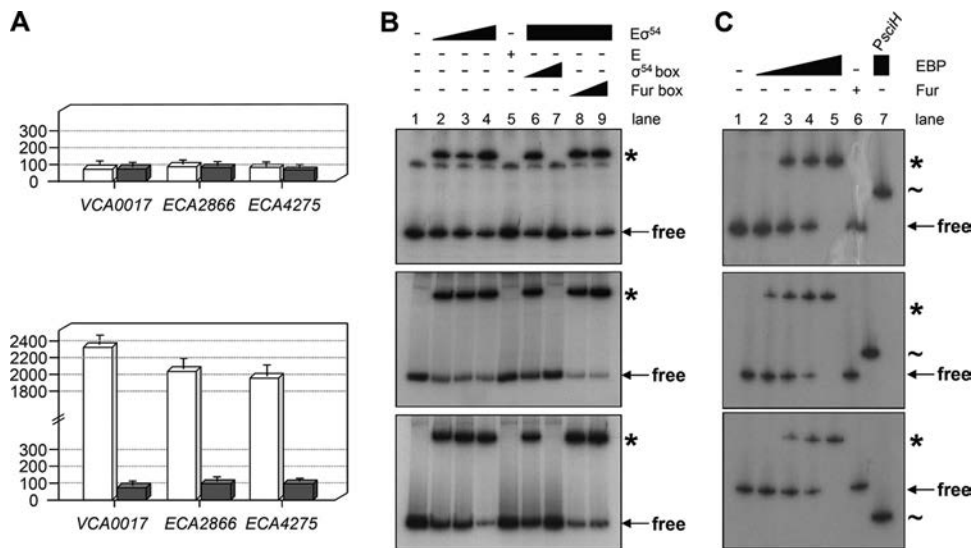


FIG. 6. $E\sigma^{54}$ and bEBP bind and regulate orphan *hcp* and *vgrG* genes. (A) β -Glucuronidase activity of the indicated transcriptional fusions in the wild-type strain (white bars) and its *rpoN* derivative strains (black bars), in the absence (upper graph) or presence (lower graph) of bEBP overproduction. (B and C) Gel shift assays with the σ^{54} -RNA polymerase complex (B) or purified bEBP (C) and the *V. cholerae hcp-vgrG* ($P_{VCA0017}$; upper panel) or the *Pectobacterium atrosepticum hcp-vgrG* ($P_{ECA2866}$; middle panel; $P_{ECA4275}$; lower panel) promoters. See legends to Fig. 3 and 5 for details. Retarded complexes are indicated by asterisks.

of both operons is σ^{54} and bEBP dependent. However, we have not yet mapped the bEBP binding UAS sequences in the promoter regions. These *cis*-acting sequences are often composed of palindromes. Several palindromes can be identified by bioinformatic analyses in these promoters but remain to be experimentally validated by DNase footprint experiments. Interestingly, the identified palindromes do not share sequence similarities, a result which is supported by the differences observed for the helix-turn-helix DNA binding domains of the T6SS-associated bEBPs (see Fig. S2 in the supplemental material). Interaction between $E\sigma^{54}$ and bEBPs is usually facilitated by the action of DNA bending proteins, such as IHF (62). Here again, IHF binding sequences can be identified by *in silico* approaches, but it remains to be seen how IHF contributes to T6SS regulation.

Our results also showed that $E\sigma^{54}$ and bEBP bind and regulate the expression of orphan *hcp* and *vgrG* genes, demonstrating that the core T6SS cluster and accessory elements are coregulated. It is noteworthy that only few proteins secreted by T6SS have been identified. One may hypothesize that the expression of genes encoding protein substrates is coregulated with the main cluster. Indeed, the toxin/antitoxin system secreted through the *P. aeruginosa* HSI-1 T6SS has been recently shown to be coregulated with the HSI-1 gene cluster (21). Proteome or transcriptome analyses of cells overproducing the enhancer binding protein may thus help to identify coregulated genes and hence putative protein substrates.

Interestingly, the observation that core T6SS gene clusters and orphan *hcp* and *vgrG* genes are coregulated raises a new question: do additional specific regulatory mechanisms modulate the expression of the orphan genes to specifically express *hcp-vgrG* pairs in certain conditions, allowing the bacteria to surface-expose distinct Hcp/VgrG structures, or does a strict coregulation allow the formation of a cocktail of Hcp/VgrG

structures? This is an exciting question that will require further investigation.

One striking observation from this study is that σ^{54} -regulated T6SS gene clusters include those of plant pathogen bacteria (*Pectobacterium atrosepticum*, *Pseudomonas syringae* pv. tomato), marine bacteria (*Marinomonas* sp.), or human environmental pathogens (such as *V. cholerae* and *A. hydrophila*, which are ubiquitous inhabitants of brackish water). None of the strict animal or human pathogens, such as pathogenic *E. coli* or *Salmonella*, have σ^{54} -dependent promoters, an observation which suggests that this regulatory mechanism has been hijacked by environmental strains. However, the different T6SS gene clusters used in this study are not phylogenetically related (6, 9), suggesting that the σ^{54} -dependent regulation results from a distinct evolution history for adaptation to a specific niche rather than acquisition by horizontal gene transfer. In Gram-negative bacteria, σ^{54} controls the expression of a number of genes and operons, essentially those involved in nitrogen metabolism or assimilation (44). These genes are induced in nitrogen starvation, a condition which is also found in the soil, in water, or in the plant rhizosphere. One may hypothesize that environmental strains have rerouted a physiological regulatory mechanism to spatially and temporarily induce the expression of virulence or adaptation genes, such as those encoding T6SS. Because of the role of T6SS in bacteria/bacteria interactions (21, 30, 46, 47), it is tempting to hypothesize that this regulatory mechanism may help bacterial colonization in a highly competitive environment, such as the rhizosphere.

A bioinformatic survey of putative σ^{54} -dependent T6SS gene clusters suggests that several other clusters might be regulated by σ^{54} , including those of *P. syringae* pv. tomato and *P. aeruginosa* HSI-2 and HSI-3. Other virulence genes of plant pathogens, such as the phytotoxin coronatine, the alginate biosyn-

thesis pathway, and the type III secretion system in *P. syringae*, are regulated by σ^{54} (1, 18, 37). Regarding *P. aeruginosa*, although this bacterium is an opportunistic human pathogen, it has also been shown to be involved in plant pathogenesis. Interestingly, the HSI-1 cluster is regulated by the RetS/LadS sensor kinase pair and the GacS/GacA/Rsm pathway, a regulatory mechanism allowing the bacteria to switch from the acute phase of infection to the chronic phase (36). Furthermore, high-throughput screens have shown that the HSI-1 cluster, but not the HSI-2 and HSI-3 clusters, is required for lung infection in a rat model of respiratory infection, and antibodies against the HSI-1-linked Hcp protein are found in the sputum of cystic fibrosis patients infected with *P. aeruginosa* (36, 39). However, recent studies showed that the HSI-1 machinery translocates antibacterial effectors (21). These data suggest that the HSI-1 T6SS might serve as interbacterial competition in animals or humans, whereas our observation suggests that the HSI-2 and HSI-3 T6SS might have importance in the environment or for the development of plant diseases. Indeed, σ^{54} is required for full virulence toward plant models in *P. syringae* and *P. aeruginosa* (1, 19, 20, 37) and a redundant role of *P. aeruginosa* HSI-2 and HSI-3 T6SS in virulence toward the plant model *Arabidopsis thaliana* has been reported (26). The survey also shows that a putative σ^{54} -dependent promoter is found upstream of the *Yersinia pseudotuberculosis* and *Yersinia pestis* T6SS gene clusters. Here again, these animal and human pathogens have a infection cycle involving development in the flea, and one might suggest that these T6SS are likely involved in flea colonization rather than development of symptoms in humans. This prediction is strengthened by transcription profiling studies showing that the *Y. pestis* cluster is induced at low temperatures and repressed at 37°C, suggesting a role in the flea vector (35).

Most of the clusters studied in this work, such as *V. cholerae*, *A. hydrophila*, and *Pectobacterium atrosepticum*, but also *Pseudomonas aeruginosa* HSI-2 and HSI-3, have also been shown to be regulated by quorum-sensing or two-component systems (5, 22, 25, 26, 28). This raises the question of how these different regulatory mechanisms are coordinated. Zheng et al. recently suggested an elegant model to explain how the *V. cholerae* *vas* cluster is regulated, by coordinating the action of the quorum-sensing, histone-like TsrA, and σ^{54} pathways (67). Although we have demonstrated the existence of σ^{54} promoters, it remains possible that additional σ^{70} promoters might be responsive to other regulatory elements. One alternate hypothesis is that bEBP phosphorylation occurs through a quorum-sensing-dependent signaling pathway or is dependent upon a sensor kinase and a specific environmental signal which remain to be identified. Interestingly, the *Pectobacterium atrosepticum* T6SS gene cluster has been shown to be induced by cell extracts of potato tubers (31, 32), suggesting that the bacteria sense specific compounds and provide an output response leading to T6SS expression. In all these cases, defining the interplay or convergence of different regulatory pathways is essential to an understanding of the complete network of regulation.

ACKNOWLEDGMENTS

We are grateful to Andrew Johnston and Andrew Curson (University of East Anglia, Norwich, United Kingdom) for strain *Marinomonas* sp. MWYL-1, Béatrice Py (Institut de Microbiologie de la Méditer-

ranée [IMM], Marseille, France) for strain *Pectobacterium carotovorum* ssp. *atrosepticum* SCRI193, Guy Condemine (Institut Nationale des Sciences Appliquées [INSA], Lyon, France) for strain *P. carotovorum* ssp. *atrosepticum* SCRI1043, Peter S. Howard (University of Saskatchewan, Canada) for strain *Aeromonas hydrophila* ATCC7966, Anne Delcour (University of Houston, Houston, TX) for strain *Vibrio cholerae* O395, Mireille Ansaldi for plasmids, protocols, and discussions, Laurent Loiseau for plasmid pUIDC1 and protocols, Patrice Moreau for strains, and Emmanuelle Bouveret and members of the Llobès, Bouveret, and Sturgis research groups for discussions, helpful comments, and encouragement. We thank the three anonymous reviewers for their interesting comments and suggestions. We thank Ginette O'yélaivécé for encouragement.

This work is supported by the Institut National des Sciences Biologiques of the Centre National de la Recherche Scientifique through a Projet Exploratoire—Premier Soutien (PEPS) grant (SDV.2009-1935) and an Agence National de la Recherche grant (ANR-10-JCJC-1303-03) to E.C. Y.R.B is supported by a graduate doctoral fellowship from the French Ministry of Research.

REFERENCES

- Alarcón-Chaidez, F. J., L. Keith, Y. Zhao, and C. L. Bender. 2003. RpoN (σ^{54}) is required for plasmid-encoded coronatine biosynthesis in *Pseudomonas syringae*. *Plasmid* **49**:106–117.
- Aschtgen, M. S., C. S. Bernard, S. de Bentzmann, R. Llobès, and E. Cascales. 2008. SciN is an outer membrane lipoprotein required for type VI secretion in enteroaggregative *Escherichia coli*. *J. Bacteriol.* **190**:7523–7531.
- Aschtgen, M. S., M. Gavioli, A. Dessen, R. Llobès, and E. Cascales. 2010. The SciZ protein anchors the enteroaggregative *Escherichia coli* type VI secretion system to the cell wall. *Mol. Microbiol.* **75**:886–899.
- Baba, T., et al. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**:2006.0008.
- Bernard, C. S., Y. R. Brunet, E. Gueguen, and E. Cascales. 2010. Nooks and crannies in type VI secretion regulation. *J. Bacteriol.* **192**:3850–3860.
- Bingle, L. E., C. M. Bailey, and M. J. Pallen. 2008. Type VI secretion: a beginner's guide. *Curr. Opin. Microbiol.* **11**:3–8.
- Bladergroen, M. R., K. Badelt, and H. P. Spink. 2003. Infection-blocking genes of a symbiotic *Rhizobium leguminosarum* strain that are involved in temperature-dependent protein secretion. *Mol. Plant Microbe Interact.* **16**:53–64.
- Bordes, P., et al. 2004. Communication between Esigma(54), promoter DNA and the conserved threonine residue in the GAFTGA motif of the PspF sigma-dependent activator during transcription activation. *Mol. Microbiol.* **54**:489–506.
- Boyer, F., G. Fichant, J. Berthod, Y. Vandenbrouck, and I. Attree. 2009. Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be learned from available microbial genomic resources? *BMC Genomics* **10**:104.
- Buck, M., M. T. Gallegos, D. J. Studholme, Y. Guo, and J. D. Gralla. 2000. The bacterial enhancer-dependent σ^{54} (σ^N) transcription factor. *J. Bacteriol.* **182**:4129–4136.
- Casaz, P., M. T. Gallegos, and M. Buck. 1999. Systematic analysis of sigma54 N-terminal sequences identifies regions involved in positive and negative regulation of transcription. *J. Mol. Biol.* **292**:229–239.
- Cascales, E. 2008. The type VI secretion toolkit. *EMBO Rep.* **9**:735–741.
- Chen, P., and L. J. Reitzer. 1995. Active contribution of two domains to cooperative DNA binding of the enhancer-binding protein nitrogen regulator I (NtrC) of *Escherichia coli*: stimulation by phosphorylation and the binding of ATP. *J. Bacteriol.* **177**:2490–2496.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U. S. A.* **97**:6640–6645.
- Dudley, E. G., N. R. Thomson, J. Parkhill, N. P. Morin, and J. P. Nataro. 2006. Proteomic and microarray characterization of the AggR regulon identifies a *phoU* pathogenicity island in enteroaggregative *Escherichia coli*. *Mol. Microbiol.* **61**:1267–1282.
- Feng, J., et al. 1992. Role of phosphorylated metabolic intermediates in the regulation of glutamine synthetase synthesis in *Escherichia coli*. *J. Bacteriol.* **174**:6061–6070.
- Filloux, A., A. Hachani, and S. Bleves. 2008. The bacterial type VI secretion machine: yet another player for protein transport across membranes. *Microbiology* **154**:1570–1583.
- Hendrickson, E. L., P. Guevera, and F. M. Ausubel. 2000. The alternative sigma factor RpoN is required for *hnp* activity in *Pseudomonas syringae* pv. *maculicola* and acts at the level of *hnpL* transcription. *J. Bacteriol.* **182**:3508–3516.
- Hendrickson, E. L., et al. 2000. Virulence of the phytopathogen *Pseudomonas syringae* pv. *maculicola* is RpoN dependent. *J. Bacteriol.* **182**:3498–3507.
- Hendrickson, E. L., J. Plotnikova, S. Mahajan-Miklos, L. G. Rahme, and F. M. Ausubel. 2001. Differential roles of the *Pseudomonas aeruginosa* PA14

- rpoN* gene in pathogenicity in plants, nematodes, insects, and mice. *J. Bacteriol.* **183**:7126–7134.
21. Hood, R. D., et al. 2010. A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell Host Microbe* **7**:25–37.
 22. Ishikawa, T., P. K. Rompikuntal, B. Lindmark, D. L. Milton, and S. N. Wai. 2009. Quorum sensing regulation of the two *hcp* alleles in *Vibrio cholerae* O1 strains. *PLoS One* **4**:e6734.
 23. Jefferson, R. A., S. M. Burgess, and D. Hirsh. 1986. β -Glucuronidase from *Escherichia coli* as a gene-fusion marker. *Proc. Natl. Acad. Sci. U. S. A.* **83**:8447–8451.
 24. Jyot, J., N. Dasgupta, and R. Ramphal. 2002. FleQ, the major flagellar gene regulator in *Pseudomonas aeruginosa*, binds to enhancer sites located either upstream or atypically downstream of the RpoN binding site. *J. Bacteriol.* **184**:5251–5260.
 25. Khajanchi, B. K., et al. 2009. *N*-Acylhomoserine lactones involved in quorum sensing control the type VI secretion system, biofilm formation, protease production, and *in vivo* virulence in a clinical isolate of *Aeromonas hydrophila*. *Microbiology* **155**:3518–3531.
 26. Lesic, B., M. Starkey, J. He, R. Hazan, and L. G. Rahme. 2009. Quorum sensing differentially regulates *Pseudomonas aeruginosa* type VI secretion locus I and homologous loci II and III, which are required for pathogenesis. *Microbiology* **155**:2845–2855.
 27. Leung, K. Y., B. A. Siame, H. Snowball, and Y. K. Mok. 2011. Type VI secretion regulation: crosstalk and intracellular communication. *Curr. Opin. Microbiol.* **14**:9–15.
 28. Liu, H., et al. 2008. Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen *Pectobacterium atrosepticum*. *PLoS Pathog.* **4**:e1000093.
 29. Lukat, G. S., W. R. McCleary, A. M. Stock, and J. B. Stock. 1992. Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors. *Proc. Natl. Acad. Sci. U. S. A.* **89**:718–722.
 30. MacIntyre, D. L., S. T. Miyata, M. Kitaoka, and S. Pukatzki. 2010. The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. *Proc. Natl. Acad. Sci. U. S. A.* **107**:19520–19524.
 31. Mattinen, L., R. Nissinen, T. Riipi, N. Kalkkinen, and M. Pirhonen. 2007. Host-extract induced changes in the secretome of the plant pathogenic bacterium *Pectobacterium atrosepticum*. *Proteomics* **7**:3527–3537.
 32. Mattinen, L., et al. 2008. Microarray profiling of host-extract-induced genes and characterization of the type VI secretion cluster in the potato pathogen *Pectobacterium atrosepticum*. *Microbiology* **154**:2387–2396.
 33. McCleary, W. R., and J. B. Stock. 1994. Acetyl phosphate and the activation of two-component response regulators. *J. Biol. Chem.* **269**:31567–31572.
 34. Miyata, S. T., et al. The *Vibrio cholerae* type VI secretion system: evaluating its role in the human disease cholera. *Front. Microbiol.* **1**:117.
 35. Motin, V. L., et al. 2004. Temporal global changes in gene expression during temperature transition in *Yersinia pestis*. *J. Bacteriol.* **186**:6298–6305.
 36. Mougous, J. D., et al. 2006. A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* **312**:1526–1530.
 37. Peñaloza-Vázquez, A., M. K. Fakhr, A. M. Bailey, and C. L. Bender. 2004. AlgR functions in *algC* expression and virulence in *Pseudomonas syringae* pv. *syringae*. *Microbiology* **150**:2727–2737.
 38. Persson, O. P., et al. 2009. High abundance of virulence gene homologues in marine bacteria. *Environ. Microbiol.* **11**:1348–1357.
 39. Potvin, E., et al. 2003. *In vivo* functional genomics of *Pseudomonas aeruginosa* for high-throughput screening of new virulence factors and antibacterial targets. *Environ. Microbiol.* **5**:1294–1308.
 40. Pukatzki, S., et al. 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc. Natl. Acad. Sci. U. S. A.* **103**:1528–1533.
 41. Pukatzki, S., S. B. McAuley, and S. T. Miyata. 2009. The type VI secretion system: translocation of effectors and effector-domains. *Curr. Opin. Microbiol.* **12**:11–17.
 42. Rao, P. S., Y. Yamada, Y. P. Tan, and K. Y. Leung. 2004. Use of proteomics to identify novel virulence determinants that are required for *Edwardsiella tarda* pathogenesis. *Mol. Microbiol.* **53**:573–586.
 43. Rappas, M., D. Bose, and X. Zhang. 2007. Bacterial enhancer-binding proteins: unlocking sigma54-dependent gene transcription. *Curr. Opin. Struct. Biol.* **17**:110–116.
 44. Reitzer, L., and B. L. Schneider. 2001. Metabolic context and possible physiological themes of sigma(54)-dependent genes in *Escherichia coli*. *Microbiol. Mol. Biol. Rev.* **65**:422–444.
 45. Schell, M. A., et al. 2007. Type VI secretion is a major virulence determinant in *Burkholderia mallei*. *Mol. Microbiol.* **64**:1466–1485.
 46. Schwarz, S., R. D. Hood, and J. D. Mougous. 2010. What is type VI secretion doing in all those bugs? *Trends Microbiol.* **18**:531–537.
 47. Schwarz, S., et al. 2010. *Burkholderia* type VI secretion systems have distinct roles in eukaryotic and bacterial cell interactions. *PLoS Pathog.* **6**:e1001068.
 48. Shalom, G., J. G. Shaw, and M. S. Thomas. 2007. *In vivo* expression technology identifies a type VI secretion system locus in *Burkholderia pseudomallei* that is induced upon invasion of macrophages. *Microbiology* **153**:2689–2699.
 49. Studholme, D. J., and R. Dixon. 2003. Domain architectures of sigma54-dependent transcriptional activators. *J. Bacteriol.* **185**:1757–1767.
 50. Studholme, D. J., S. R. Wigneshweraraj, M. T. Gallegos, and M. Buck. 2000. Functionality of purified σ^N (σ^{54}) and a NifA-like protein from the hyperthermophile *Aquifex aeolicus*. *J. Bacteriol.* **182**:1616–1623.
 51. Suarez, G., et al. 2008. Molecular characterization of a functional type VI secretion system from a clinical isolate of *Aeromonas hydrophila*. *Microb. Pathog.* **44**:344–361.
 52. Suarez, G., J. C. Sierra, M. L. Kirtley, and A. K. Chopra. 2010. Role of a type 6 secretion system effector Hcp of *Aeromonas hydrophila* in modulating activation of host immune cells. *Microbiology* **156**:3678–3688.
 53. Syed, K. A., et al. 2009. The *Vibrio cholerae* flagellar regulatory hierarchy controls expression of virulence factors. *J. Bacteriol.* **191**:6555–6570.
 54. Tabor, S., and C. C. Richardson. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled expression of specific genes. *Proc. Natl. Acad. Sci. U. S. A.* **82**:1074–1078.
 55. Tardat, B., and D. Touati. 1993. Iron and oxygen regulation of *Escherichia coli* MnSOD expression: competition between the global regulators Fur and ArcA for binding to DNA. *Mol. Microbiol.* **9**:53–63.
 56. Tintut, Y., C. Wong, Y. Jiang, M. Hsieh, and J. D. Gralla. 1994. RNA polymerase binding using a strongly acidic hydrophobic-repeat region of sigma 54. *Proc. Natl. Acad. Sci. U. S. A.* **91**:2120–2124.
 57. Tropel, D., and J. R. van der Meer. 2002. Identification and physical characterization of the HbpR binding sites of the *hbpC* and *hbpD* promoters. *J. Bacteriol.* **184**:2914–2924.
 58. van den Ent, F., and J. Löwe. 2006. RF cloning: a restriction-free method for inserting target genes into plasmids. *J. Biochem. Methods* **67**:67–74.
 59. Vieira, J., and J. Messing. 1991. New pUC-derived cloning vectors with different selectable markers and DNA replication origins. *Gene* **100**:189–194.
 60. Weber, B., M. Hasic, C. Chen, S. N. Wai, and D. L. Milton. 2009. Type VI secretion modulates quorum sensing and stress response in *Vibrio anguillarum*. *Environ. Microbiol.* **11**:3018–3028.
 61. Weiss, V., and B. Magasanik. 1988. Phosphorylation of nitrogen regulator I (NRI) of *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **85**:8919–8923.
 62. Wigneshweraraj, S. R., et al. 2008. Modus operandi of the bacterial RNA polymerase containing the sigma54 promoter-specificity factor. *Mol. Microbiol.* **68**:538–546.
 63. Wigneshweraraj, S. R., P. Casaz, and M. Buck. 2002. Correlating protein footprinting with mutational analysis in the bacterial transcription factor sigma54 (sigmaN). *Nucleic Acids Res.* **30**:1016–1028.
 64. Zhang, X., et al. 2002. Mechanochemical ATPases and transcriptional activation. *Mol. Microbiol.* **45**:895–903.
 65. Zhang, N., et al. 2009. The role of the conserved phenylalanine in the sigma54-interacting GAFTGA motif of bacterial enhancer binding proteins. *Nucleic Acids Res.* **37**:5981–5992.
 66. Zheng, J., and K. Y. Leung. 2007. Dissection of a type VI secretion system in *Edwardsiella tarda*. *Mol. Microbiol.* **66**:1192–1206.
 67. Zheng, J., O. S. Shin, D. E. Cameron, and J. J. Mekalanos. 2010. Quorum sensing and a global regulator TsrA control expression of type VI secretion and virulence in *Vibrio cholerae*. *Proc. Natl. Acad. Sci. U. S. A.* **107**:21128–21133.